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14. ABSTRACT

The androgen receptor (AR) signaling-pathway plays crucial roles in the growth and progression of prostate cancer cells. Recent studies indicate that β-Catenin physically binds to AR and enhances its transcripitional activity in a ligand dependent manner. p53 has also been implicated in AR signaling because of its ability to induce expression of Siah1, which binds and activates E3 ligase complexes which degrade β-Catenin. In this study, we demonstrated the biological significance and molecular mechanisms by which AR is regulated by the p53-induced Siah1 protein. Moreover, we identified the relevant proteins that are targeted for degradation by Siah1 besides β-Catenin. Thus, enhanced Siah function may suppress the ability of androgen to promote tumor cell growth. Understanding more about the functions of Siah-family proteins may therefore suggest novel strategies for chemoprevention and for improved treatment of prostate cancer.

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W81XWH-05-1-0007: The role of Siah1-induced degradation of β-Catenin in androgen receptor.

Shu-ichi Matsuzawa Ph.D.

Introduction:

The androgen receptor (AR) signaling-pathway plays crucial roles in the growth and progression of prostate cancer cells. Recent studies indicate that β-Catenin physically binds to AR and enhances its transcripitional activity in a ligand dependent manner. Mutations that inactivate the p53 gene occur in one-third to half of all human prostate cancers and have been correlated with shorter patient survival. Loss of p53 is known to render tumor cells more resistant to a wide range of anticancer drugs and radiation [1, 2]. It would be highly desirable therefore to have a means of functionally restoring p53 activity in prostate cancers in which this gene has become inactivated. A strategy for accomplishing this is to identify the downstream effectors of p53's actions and to find ways of enhancing their activity in the absence of p53. The human Siah gene is localized on chromosome 16q12-13, a region reported to contain a candidate tumor suppressor gene in various types of cancer, including prostate cancer. Our previous results indicate that over-expression of the Siah1 protein can induce either growth arrest or apoptosis, depending on the cell line tested [3]. The findings suggest that p53-mediated induction of Siah1 expression could play an important role in the mechanisms by which this tumor suppressor inhibits cell proliferation and induces apoptosis. In this study, we designed experiments to test the hypothesis that Siah1 is an important mediator of p53's effects in prostate cancers. If the hypothesis proves to be correct, then the results derived from these studies will lay the groundwork for development of new strategies for restoring p53-like functions in prostate cancers, thus improving therapeutic outcomes for men with carcinoma of the prostate. Our preliminary data also suggest a model by which Siah-family proteins can regulate AR signaling through ubiquitation and degradation of β-Catenin. The central goal of this proposal is to test the validity of this hypothesize model, linking it ultimately to the regulation of tumor cell growth. However, at this point, essentially nothing is known about the relative importance of Siah1 for p53 responses compared to AR target genes. We therefore proposed to address the following questions:

1. Is Siah1 a p53 primary response gene in the prostate?
2. What are the mechanisms by which Siah1 inhibits AR activity?
3. Does Siah1 control the sensitivity of human prostate cancer cell lines to anti-androgens in vitro and in xenograph mouse models?
4. What are the relevant proteins that are targeted for degradation by Siah1 besides β-Catenin?

Body:
We have made excellent progress in the first year of renewed funding towards accomplishing these aims. A brief summary follows:

<u>Aim #1.</u> Examine the effects of p53 on expression of the *Siah1* gene in prostate cells. The functional p53 binding site was identified in intron 1 of the Siah1 gene.
Using a computer algorithm, we identified nine potential p53-response elements (REs) in intron 1 of the *Siah1* gene (Figure 1A). To explore the possibility that Siah1 is a p53-regulated gene, we cloned four fragments of intron 1 of the *Siah1* gene into a luciferase reporter gene plasmid (named pGL3E-Siah1p53RE-1, 2, 3, and 4) and used transient transfection reporter gene assays to study the effects of p53 on its activity. PPC1, a p53-null prostate cancer cell line was transfected with each of the fragments with or without a plasmid containing wild-type p53. Among the four fragments, only p53RE-3 showed increased luciferase activity in response to p53 (Figure 1B). Since p53RE-3 still includes the potential p53 REs, we made three constructs including each of three p53 REs (named p53RE-3-1, 3-2, and 3-3) and performed luciferase assays (Figure 1C). Among these fragments, only p53RE-3-2 showed increased luciferase activity (Figure 1D). To examine this result, a vector was constructed with four point mutations in the potential p53 binding sequence (Figure 1E) and luciferase activity was assayed. As expected, the mutant did not respond to p53 (Figure 1F). We concluded that the functional p53 RE is located in the region p53RE3-2 of the Siah1 gene intron 1.

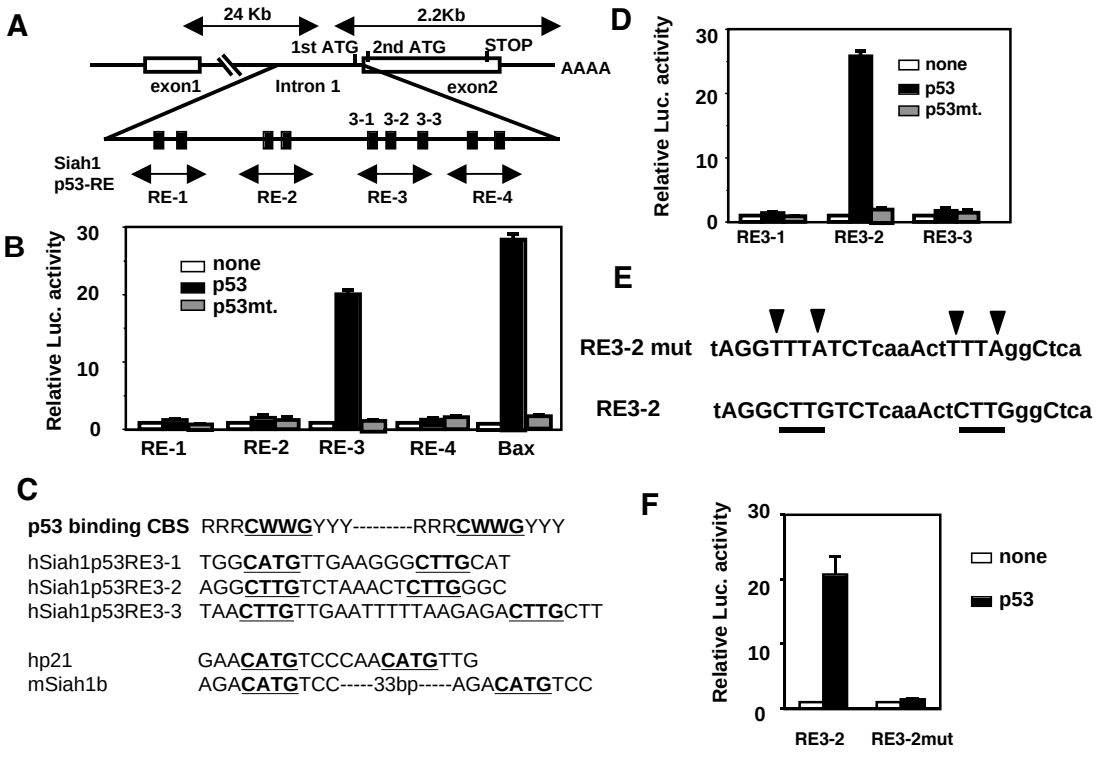

Figure 1. Transcriptional activation by p53 was identified in the intron 1 of the *Siah1* gene. (**A**) Mapping of potential p53 responsive elements (REs) in intron 1 of the *Siah1* gene. Potential p53 binding sites are shown by closed boxes. Lower arrows show the region of various inserts cloned into pGL3-enhancer (pGL3E) vector. The numbers show the name of pGL3E-Siah1p53RE vector. (**B**) Luciferase assay. PPC1 cells were transfected with the same amounts of the different pGL3E-Siah1 promoter vectors (pGL3E-Siah1p53RE-1, 2, 3, and 4), pCMV–p53wt, pCMV–p53mut, and pCMV-β-gal as a transfection efficiency control. pGL3E-Bax vector was used as a positive control. Luciferase activity was measured in cell lysates 24 hrs later and the data were normalized relative to β-galactosidase (mean ± SD; n=3). Lipofectamine™ 2000 (Invitrogen) was used for transfection. The same amounts of plasmid DNA was kept by the addition of empty expression vector. (**C**) Sequences of representative p53-inducible genes and three potential p53REs of the *Siah1* gene inserted into pGL3E-Siah1p53RE3-1, 3-2, and 3-3 vector. The consensus p53 binding sequence (CBS) is show above. (**D**) Luciferase assay using pGL3E-Siah1p53RE3-1, 3-2, and 3-3 promoter vectors. Luciferase activity was measured as described in (C). (**E**) The sequences of potential p53RE in the pGL3E-Siah1p53RE3-2 vector. Mutated points (pGL3E-Siah1p53RE3-2mut) are shown above. (**F**) Luciferase assay using pGL3E-Siah1p53RE3-2 and 3-2mut promoter vectors. Luciferase activity was measured as described in (C).

To further investigate whether the p53 protein is actually able to bind this p53 RE, Electrophoretic Mobility-Shift Assay (EMSA) (Figure 2A) and Chromatin Immunoprecipitation (ChIP) assays (Figure 2B) were performed using MCF7 cells (Figure 2A). In both experiments, we observed actual p53 binding to the p53-RE-3-2. Thus, we concluded that p53 binds directly to the identified p53 RE of Siah1 *in vitro* and *in vivo*.

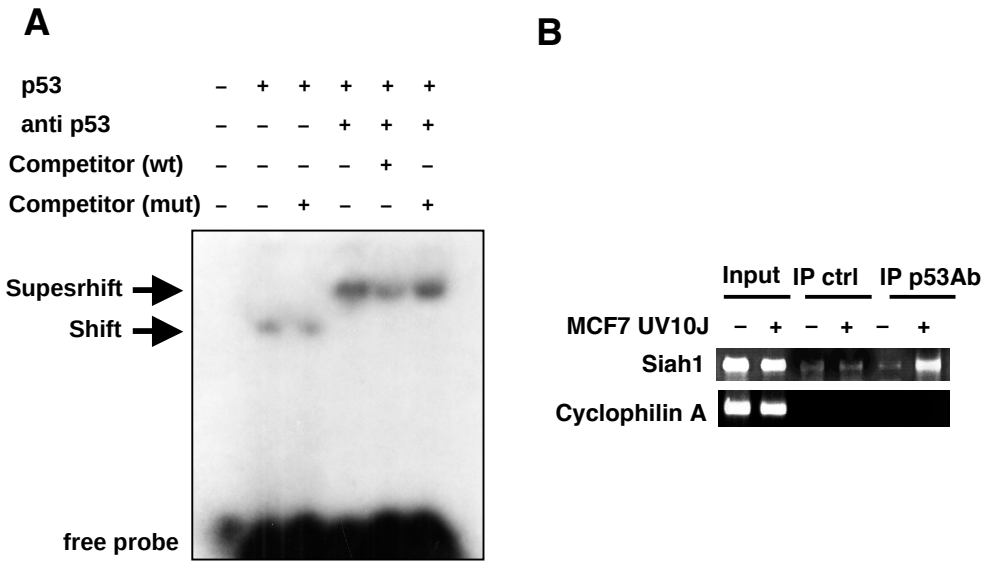

Figure 2. p53 binds directly to the p53RE of *Siah-1 in vitro* and *in vivo*. (**A**) Electrophoretic Mobility-Shift Assay was performed using a 27bp probe including the p53 binding site of *Siah1* (Shown in Figure 2e) and recombinant p53 protein (30 ng, Active Motif) in the presence or absence of anti-p53 antibody (Pab421, Oncogene). Specific binding was determined by adding either unlabeled homologuos probe DNA or mutant DNA (Shown in Figure 2e) at 50-fold molar excess. The positions of the free probe and shifted bands are indicated. (**B**) Chromatin Immunoprecipitation assay was performed as described previously (de Belle, I et al. 2000, BioTechniques). Chromatins from MCF7 cells with or without 24hr after UV-irradiation (10 J/m^2) were immunoprecipitated with (IP p53) or without (IP ctrl) anti-p53 antibody (FL-393, Santa Cruz) overnight at 4°C and followed by incubation with protein A-Sepharose beads (Santa Cruz) for

an additional 1hr. After the DNA fragments were purified, PCR amplification was performed using the Siah1 specific primers (5'-AGACATAGCTCATTGCAGCCTTTAC-3' and 5'-TATTTTGAGGCTTCCACCCAAGC-3') designed to amplify a 280-bp fragment including p53 binding site. The same samples were used for PCR using Cyclophilin A primers (5'-CTCCTTTGAGCTGTTTGCAG-3' and 5'-CACCACATGCTTGCCATCC-3') as a negative control. Total lysate was used as a positive control (input).

Next, to investigate whether Siah1 protein is actually expressed by p53 in cells, RNase protection assays and western bolt analysis were performed using HEK293 cells. Results showed no difference of expression of Siah1 mRNA before and after when HEK293 cells were over-expressed with p53 (Figure 3A). However, a larger size mRNA band was highly expressed after these cells were over-expressed with p53 (especially 12 hours later). Expression of the long type of Siah1 protein, named Siah1L, was also confirmed by western blotting using Siah1-specific antibodies (Figure 3B). The protein sizes of endogenous Siah1 and Siah1L corresponded with transiently transfected Siah1 and Siah1L, respectively using expression vectors. The Siah1L expression was confirmed using the Siah1L specific short interfering RNA (siRNA) vector (pSuppress-Siah1L) (Figure 3C). The protein level of Siah1L was decreased by pSuppress-Siah1L, whereas that of Siah1 did not change. These results indicate that only Siah1L is upregulated in response to p53. Currently, we are analyzing other types of prostate cancer cell lines including LNCaP, PC3, ALVA31, Du145, JCA-1, Tsu-prl and the immortalized prostate epithelial cell line 267β1 cells to contrast the expression of Siah1 and β-Catenin proteins.

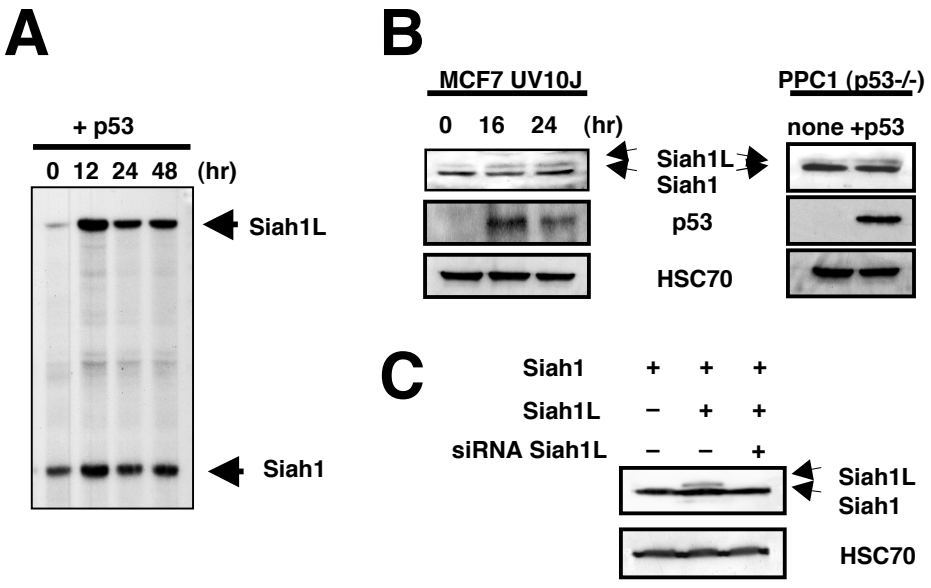

Figure 3. (A) RNase protection assay of *Siah1* mRNA after p53 over-expression. HEK293 cells were transiently transfected with 10 μg of pCMV-p53wt. Total RNAs were extracted from cells at 0, 12, 24 and 48 hr after transfection and *Siah1* RNA expression was measured using a probe containing 324 bp of *Siah1* cDNA. RNase protection assay was performed as previously described [3]. (B) Left; MCF7 cells were treated with UV-irradiation (10 J/m^2). Total proteins were extracted from cells at 0, 16, 24 hr after transfection. Right; PPC1 cells were transiently transfected with empty vector or pCMV-p53wt. Cells were lysed with RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS). The same amounts of cells lysates (60 μg per lane) were analyzed by immunoblotting, using antibodies specific for Siah1 (N-15, Santa Cruz), p53 (Pab421, Oncogene). The membrane was reprobed with goat anti-HSC70

antibody (K-19, Santa Cruz) as a control. (**C**) PPC1 cells were transiently transfected with pcDNA3-Siah1, pcDNA3-Siah1L, and pSuppress-Siah1L (siRNA-Siah1L) in various combinations, as indicated. Empty pcDNA3 and pSuppress vectors were used as controls. After 24 hrs, the lysates (60 μg per lane) were analyzed by immunoblotting, using antibodies specific for Siah1. The membrane was reprobed with goat anti-HSC70 antibody as a control. The cDNAs encoding human *Siah1* and *Siah1L* were generated by PCR as described previously [3]. pSuppress, an siRNA-expressing plasmid, was kindly provided by D. Billadeau (Mayo Clinic, Rochester, MN). The following *Siah1L* target sequence was inserted: 5'-CTCCTGCCTCCTTATGTAT-3'.

<u>Aim #2</u>. Determine the importance of p53-induced degradation of β-Catenin in Siah-mediated suppression of AR activity.

To determine whether the degradation of β-Catenin by Ebi is important for Siah-mediated suppression of AR activity, a dominat-negative Ebi mutant was constructed which fails to bind Skp1 but still binds to β-catenin. As expected, over-expression of the mutant Ebi abrogated the Siah1-induced suppression of AR activity, suggesting that Ebi is essential for the Siah1–induced suppression of AR activity (Figure 4A).

To further investigate whether Siah1L is necessary for p53-dependent regulation of AR activity, we used Siah1L-specific siRNA. AR activity was measured by luciferase assay using PPC1 cells transfected with pMMTV (AR responsive luciferase reporter plasmid). p53 over-expression resulted in a decrease of AR activity (Figure 4B). However, introduction of Siah1L siRNA failed to suppress AR activity by p53 (Figure 4B). These data suggest that Siah1L is important for p53-depenpent β-catenin degradation pathway.

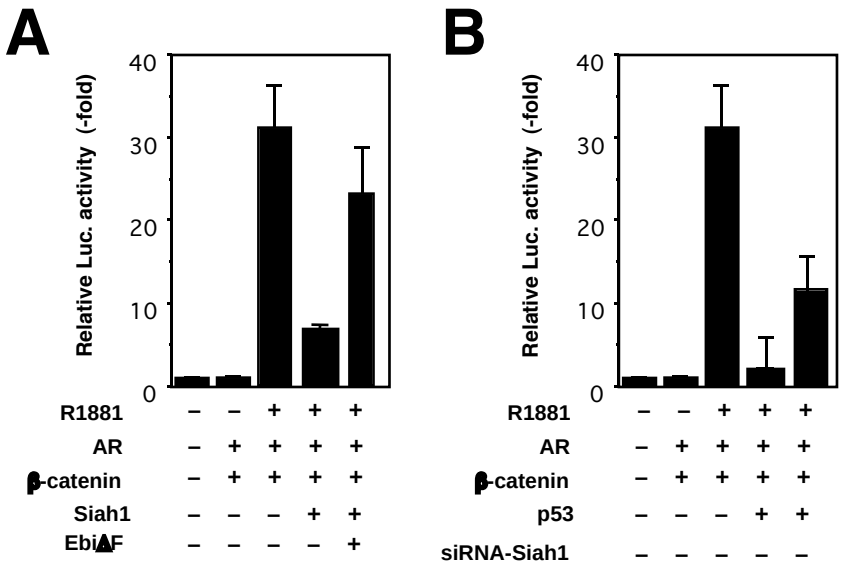

Figure 4. PPC1 cells were transiently transfected with pMMTV-Luc plasmid that contains an AR responsive element cloned upstream of a luciferase reporter gene together with pCMV-βGal as a transfection-efficiency control and the indicated plasmids encoding AR-encoding plasmid pSG5-AR(AR), β-Catenin, p53, Siah1, Ebi (EbiΔF) or pSuppress-Siah1L (siRNA-Siah1L). Twenty-four hours after transfection, cells were stimulated with 1 nM R1881. Cell extracts were prepared and assayed for luciferase and β-galactosidase activity at 48 h. Data were normalized using β-galactosidase, and results are expressed as –fold transactivation relative to cells transfected with the reporter gene alone (mean ± S.D.; n=3).

<u>Aim #3.</u> Explore the effects of Siah1 on the control of sensitivity of human prostate cancer cell lines to anti-androgens in vitro and in xenograph mouse models.
Establishment of stable cell lines with express TET-inducible Siah1 is underway.

<u>Aim #4</u>. What are the mechanisms by which Siah1 suppresses androgen receptor activity?
To identify potential targets of Siah1, yeast two-hybrid screens of cDNA libraries made from prostate cancer cell lines were performed using the human Siah1 protein as a bait. This effort has resulted in identification of a known Siah-binding protein AF4 [4, 5] and a novel protein that we call SIP2, for Siah1 Interacting Protein 2 (Figure 5). Further functional analysis of those proteins is underway.

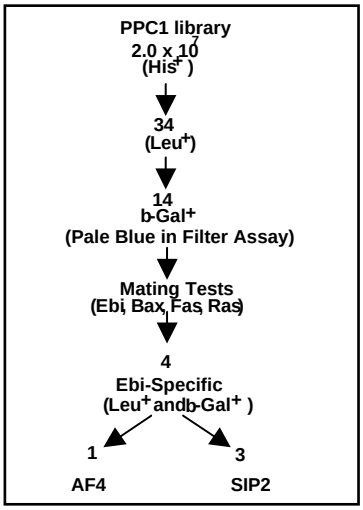

Figure 5. <u>Identification of SIP2 as a candidate Siah1-binding protein by two-hybrid cDNA library screening</u>. Library screening by the yeast two-hybrid method was performed as described [3] using pGilda encoding human Siah1 as a bait, cDNA libraries derived from PPC1, and EGY48 strain yeast. Cells were grown in either YPD medium with 1% yeast extract, 2% polypeptone, and 2% glucose, or in Burkholder's minimal medium (BMM) fortified with appropriate amino-acids as described previously [3]. Transformations were performed by a LiCl method using 0.1 mg of pJG4-5-cDNA library DNA, and 5 mg of denatured salmon sperm carrier DNA. Clones that formed on Leu-deficient BMM plates containing 2% galactose / 1% raffinose were transferred to BMM plates containing leucine and 2% glucose, and filter assays were performed for β-galactosidase measurements. The specificity of two-hybrid interactions mediated by candidate cDNA clones was evaluating by mating with RFY206 cells, which contained one of 4 different indicator pGilda plasmids encoding the following LexA bait proteins: Ebi, Bax (1-171), Fas (191-335) or v-Ras.

Key Research Accomplishment:
We have made excellent progress in the first year of funding towards accomplishing these goals. Two manuscripts are being prepared regarding Aim#1 and #2.

Reportable Outcomes:
none

Conclusions:
We have determined that *Siah1* is a primary response gene for p53. We have also demonstrated that Siah1-specific siRNA showed down-regulation of AR activity by p53. These findings suggest that Siah1 is a critical regulator of AR signaling and there are new strategies for restoring tumor suppressive pathways lost in cancers that have suffered p53 inactivation.

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Appendices:
none